Evo-devo of novel traits: the genetic basis of butterfly colour patterns

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CHAPTER 5. Innovations on butterfly wings: co-option of insect embryonic and wing patterning genes in eyespot formation

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Co-option of conserved genes and genetic pathways seems to play a fundamental role in morphological evolution. How are ancestral gene networks redeployed in the development of novel structures? How do modifications of these networks produce changes in phenotype? The eyespots of *Bicyclus anynana* butterflies provide a study system where these questions can be addressed in an integrative way. Eyespots are formed around inductive organizers called foci. Focal cells produce a morphogen the levels of which establish the colour of surrounding scales in pupal wings. The number and position of foci are determined during the last larval stage. Here we focused on several conserved genetic pathways which are fundamental to insect embryonic or wing development and are potentially redeployed in formation of eyespots. We characterized expression patterns of candidate eyespot genes and attempted to manipulate their function. Our study established a relationship between eyespot focal determination and expression of the Hox gene *Antennapedia* and suggested that this conserved transcription factor might be the first key regulator of eyespot formation. This illustrates that even highly conserved Hox genes can be co-opted to perform additional functions during animal development. Furthermore, we observed unexpected patterns of the putative eyespot morphogen *wingless* and its antisense transcripts in pupal wings and suggested their potential role for fine-tuning of gene expression. We also described unforeseen differences in the expression of Hedgehog pathway genes in *B. anynana* and *Junonia coenia*. These results suggest that the genetic mechanisms underlying eyespot formation in these butterflies might have diversified substantially.

INTRODUCTION

The origin and diversification of novel morphological traits has always fascinated biologists and laymen alike, and is now a key research theme in evolutionary developmental biology, or evo-devo (Wagner & Lynch 2009). Novelties seem to arise through co-option of conserved developmental toolkit genes (Ganfornia & Sanchez 1999; True & Carroll 2002), although recent studies also implicated taxonomically-restricted genes in the generation of lineage-specific traits (reviewed in Khalturin *et al*. 2009). The relative importance of new and conserved genes and the molecular changes that produce novelty-specific gene networks must be studied in a broad range of taxa in order to understand general principles about the evolution of novel traits.

The extremely diverse butterfly wing patterns are generated by the arrangement of pigmented scales and have no obvious homology to wing patterns of other insects. This makes them an ideal system to study the genetic and developmental mechanisms underlying the origin of novelties. Particularly eyespots have emerged as a promising evo-devo model used to investigate how novel characters arise (Keys *et al*. 1999; Brunetti *et al*. 2001; Saenko *et al*. 2008) and diversify (Allen *et al*. 2008; Beldade, French & Brakefield 2008; Monteiro 2008). Eyespots have a clear adaptive value (*e.g.* Olofsson *et al*. 2010; Robertson & Monteiro 2005) and show extreme intra- and interspecific variation in morphology (Nijhout 1991). These pattern elements are formed by the action of multiple gene networks which regulate pigment biosynthetic pathways in the developing scales (reviewed in Beldade & Saenko 2009), and changes in the organization of these networks ultimately yield the observed eyespot diversity. A more complete account of eyespot evolution awaits a detailed understanding of how ancestral gene networks are co-opted in the development of wing patterns (see Monteiro & Podlaha 2009), and of how modifications of the eyespot patterning network under the influence of natural and sexual selection produce the observed changes in the phenotype.

Until now, studies of eyespot development have focused mainly on two species of the family Nymphalidae, *Bicyclus anynana* and *Junonia coenia* (Beldade & Brakefield 2002; McMillan, Monteiro & Kapan 2002). Classical surgical manipulations have shed light on some of the cellular interactions underlying eyespot formation (Nijhout 1980; French & Brakefield 1992, 1995), and gene expression studies implicated a number of conserved genetic pathways that play essential roles in insect embryonic and wing development, and have been co-opted in eyespot formation (reviewed in Beldade & Saenko 2009). Eyespot development begins at the end of the larval stage when several wing-patterning genes are upregulated in positions of the wing epidermis corresponding to the centers, or foci, of adult eyespots. During

the early pupal stage, the cells of these foci provide positional information, in the form of one or several signaling molecules that diffuse throughout the epidermis and define cellular territories corresponding to the different colour rings of the adult eyespot. Epidermal cells respond to these morphogen(s) in a concentration-dependent manner by activating different pigment biosynthesis pathways and subsequently by producing wing scales of different colours. The Hedgehog (Hh) signaling pathway, together with the transcription factors Spalt (Sal), Engrailed (En) and Distal-less (Dll), and Notch (N) receptor, have been implicated in the process of eyespot determination (Carroll *et al*. 1994; Brakefield *et al*. 1996; Keys *et al*. 1999; Reed & Serfas 2004). The signaling molecules Wingless (Wg) and Decapentaplegic (Dpp) have both been proposed as candidate eyespot morphogens (Monteiro *et al*. 2006). All these genes were suggested by studies of their expression patterns, and functional tests of their role are yet to be performed.

Here we characterized expression patterns of key players in several conserved genetic pathways which are fundamental to insect embryonic or wing development and are potentially redeployed in different stages of eyespot formation. First, the Hh signaling pathway was implicated in eyespot focus determination in *J. coenia* larval wings, where genes encoding the Hh ligand, its receptor Patched (Ptc) and the transcription factor Cubitus interruptus (Ci) are expressed around or in eyespot foci (Keys *et al.* 1999). Based on gene expression data from this and other experiments, and on the available information about genetic interactions in *Drosophila melanogaster* wing imaginal discs, Evans and Marcus (2006) proposed two models of the genetic regulatory hierarchy for the eyespot focus determination, and suggested that several predictions of these models could be tested experimentally in *B. anynana* wild-type and Cyclops mutant butterflies (Marcus & Evans 2008). Next, the Wg pathway has been implicated in the process of focal signaling by Monteiro *et al*. (2006), who detected Wg protein in eyespot centres of early pupal wings with the antibody developed against human Wnt-1 protein. One of the known targets of Wg signaling in *Drosophila* wing imaginal discs, *Dll* (Neumann & Cohen 1997a), is expressed in the area around eyespot foci in pupal wings of *B. anynana* and other butterflies (Brakefield *et al*. 1996; Brunetti *et al*. 2001). This upregulation of *Dll* might be a response to a concentration gradient of Wg, which makes this molecule a good candidate for an eyespot morphogen that is secreted from eyespot centres and induces a cellular response in the surrounding epidermis (Neumann & Cohen 1997b). Moreover, our comparative analysis of embryonic defects in pleiotropic mutants with disturbed eyespot size and/or colour composition suggests that these mutations might have occurred in a segment polarity gene, probably a negative regulator of the Wg signaling pathway (see Chapter 3).

To test the predictions of the models proposed by Marcus & Evans, and to further investigate the role of Wg in eyespot formation, we studied the spatio-temporal expression patterns of genes encoding the Hh and Wg ligands and their respective receptors, Ptc and Frizzled (Fz), in developing wings of *B. anynana*. In addition, we examined expression patterns of the Hox genes *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*), two highly conserved transcription factors involved in arthropod embryonic patterning (Hughes & Kaufman 2002). Ubx also controls insect hindwing identity, including wing scale morphology and colour pattern elements in butterflies (Lewis *et al*. 1999; Weatherbee *et al*. 1999), but no Hox gene has been demonstrated to be involved in eyespot determination. Finally, we attempted to inhibit the functions of two transcription factors implicated in eyespot development via RNAi or morpholino injections.

MATERIAL AND METHODS

Biological material

B. anynana wild-type (WT) and mutant *Cyclops* stocks were reared in standard laboratory conditions at 27^{o}C (*cf*. Brakefield, Beldade & Zwaan 2009). Pupation times were recorded using time-lapse photography with 30 minute intervals. Larvae and pupae were anesthetized in ice-cold PBS and their wings dissected using fine foreceps and scissors. For larval wing discs, we used the staging system based on wing vein and tracheal development: stage 0 – wing disc as found after the last molt; 0.5 – vein lacunae are visible; 1 – anterior trachea extend into vein lacunae; 1.5 – posterior trachea extend into vein lacunae; 2 – most trachea reach border lacuna; 2.5 – trachea extend into border lacuna; 3 – trachea form continuous line in border lacuna (*cf*. Reed, Chen & Nijhout 2007).

Cloning of *B. anynana Antp* and *hh* homologues

Total RNA was extracted from embryos and wings dissected out of last instar larvae and 1 – 4 days old pupae. RNA extractions were done with Trizol (Invitrogen) according to manufacturer's instructions. Samples were run on an agarose gel to verify RNA quality and treated with DNase (Ambion) to remove traces of genomic DNA. For degenerate PCR, cDNA was prepared using Reverse Transcription System (Promega). The SMARTer rapid amplification of cDNA ends (RACE) Amplification Kit (Clontech) was used for cDNA preparation and 5'-RACE PCR.

To clone the *Antp* gene in *B. anynana*, protein sequences of seven insects were aligned using ClustalW (Larkin *et al*. 2007), and degenerate primers were designed in the highly conserved homeobox region (Fig. 1) using CODEHOP (Rose *et al*. 1998). A 159-bp fragment of *Antp* was amplified with primers 5'-

CAGACCCTGGAGCTGGAGAARGARTTYCAYT and 5'-GCCCTTGGTCTTGTTCTCCTTYTTCCAYTTC from embryonic cDNA using the following touchdown PCR conditions: denaturing at 95°C for 2 min; 18 cycles at 95°C for 30 s, 58 – 50°C (decreasing by 4°C per 6 cycles) for 30 s, 72°C for 1.5 min; 40 cycles at 95°C for 30 s, 48°C for 30 s, 72°C for 1.5 min; final extension at 72°C for 5 min. The product was cloned into the pGEM®T-Easy vector (Promega) and sequenced with vector primers M13F and M13R. The sequence obtained was then used to design gene-specific primers for 5'RACE PCR. The first-round PCR was performed on larval forewing cDNA with primer 5'-GATTTGGCGCTCGGTGAGACAGAGG using the following conditions: 5 cycles at 94°C for 30 s, 72°C for 3.5 min; 5 cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 3.5 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 3.5 min. This product was then used as a template in the second-round PCR with internal 5'- CCGCGTCAGGTATCGGTTGAAGTGG primer, carried out for 22 cycles (94°C for 30 s, 68°C for 30 s, 72°C for 3.5 min). The 700-bp product was excised from an agarose gel, cleaned with Wizard SV Gel and PCR Clean-Up System (Promega), cloned into the pCRII®-TOPO vector (Invitrogen), amplified and sequenced with vector primers M13F and M13R.

An available sequence of a 339-bp fragment of *B. anynana hh* homologue (Arjen van 't Hof, *pers*. *comm*.) was used to design gene-specific primers for 5'RACE PCR. The first amplification with primer 5'-GCTCCAGTGCCCACTGATGATTCTG was carried out for 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min and a final extension at 72°C for 10 min, and was followed by the second-round PCR with internal primer 5'-ACACTGATGGCGAGCGTGTTCAACT, performed under the same conditions. The ~250 bp product was cloned and sequenced in the same way as the *Antp* product, and aligned with the already available sequence. All sequences were edited in BioEdit and aligned against their insect homologues in NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). ExPASy's translation tool (http://www.expasy.ch/tools/dna.html) was used to obtain the translations of the nucleotide sequences and ClustalW (2.0.12) multiple alignment tool (http://www.ebi.ac.uk/Tools/clustalw2) to produce the protein alignments. The nucleotide sequences of *Antp* and *hedgehog* have been deposited to GenBank (HQ020406, HQ020407).

In situ hybridizations

The fragments of *B. anynana* genes encoding Wg and Hh ligands and their receptors were amplified from embryonic cDNA with the following primers: *wg* (AY218276.1) 5'-GTCATGATGCCCAATACCG/5'-GCAGTTGCATCGTTCCACTA; *fz* 5'-TGCCCATTTACACATCAGGA/5'-GTATGTGGTTCCTGGCTGCT; *ptc* (HQ020408) 5'-

CGTACTTGATGCTTGGCAGA/5'-GCCAGTAGAACGCTCAGTCC; *hh* 5'-TGACCCCTCTCGTCTTCAAC/5'-AAAACAACCAGCTCCAGTGC. The products were cloned into pCRII®-TOPO dual-promoter vector using the TOPO TA cloning kit (Invitrogen). These vectors, as well as the plasmid containing 159-bp fragment of *Antp*, were used for synthesis of *in situ* hybridization digoxygenin-labeled sense and antisense riboprobes. Plasmids were extracted from bacterial colonies with QIAprep Spin Miniprep Kit (QIAGEN) and used as templates for PCR reactions with gene-specific and vector primers M13F and M13R. The amplified products were cleaned with Wizard SV Gel and PCR Clean-Up System (Promega) and used for SP6 or T7 transcription with DIG RNA labeling mix (Roche Applied Science). All probes were run on an agarose gel and measured with NanoDrop spectrophotometer (Thermo Scientific) to assess their quality and concentration. Whole mount *in situ* hybridizations of larval and pupal wings were done according to the protocols described in Brakefield, Beldade & Zwaan (2009). Sense probes were used as negative controls.

Immunohistochemistry

Antibody stainings of larval and pupal wings were performed as described in Brakefield, Beldade & Zwaan (2009). The following antibodies were used: monoclonal mouse anti-Antp 4C3 and 8C11 (Condie, Mustard & Brower 1991) [dilution 1:50], anti-Notch C17.9C6 (Fehon *et al*. 1990) and anti-Ubx/Abd-A FP6.87 (Kelsh *et al*. 1994) [1:20], anti-En/Inv 4F11 (Patel *et al*. 1989) [1:50], polyclonal rabbit anti-Dll (Panganiban *et al.* 1995) [1:200] and anti-Sal (de Celis, Barrio & Kafatos 1999) [1:500]. Primary antibodies were detected with Alexa Fluor 488 anti-mouse and Texas Red anti-rabbit secondary antibodies (Molecular Probes) [1:200]. Images were collected on a BioRad MRC 1024 ES and Zeiss Imager M1 laser scanning confocal microscopes. For the time series experiment (Fig. 7), right wings of 70 larvae were co-stained with anti-Antp 4C3 and anti-Sal antibodies, and left wings with anti-Notch and anti-Dll antibodies.

***Antp* and *Dll* knock-down experiments**

For the production of a template for in vitro transcription, PCR with vector-specific M13F and M13R primers was performed on a pCRII®-TOPO vector containing a 450-bp *Antp* insert and the promotor sequences for the T7 and SP6 polymerases. After cleaning with Wizard SV Gel and PCR Clean-Up System (Promega), 1 µg of the PCR product was used as a template for transcription reactions with T7 and SP6 enzymes. Sense and antisense transcripts were synthesized using the MEGAscript® RNAi Kit (Ambion), according to the manufacturer's instructions, and annealed at room temperature to obtain double-stranded RNA (dsRNA). After purification in sterile 0.5xPBS, the quality of dsRNA was verified on an agarose gel, and its concentration measured with

NanoDrop spectrophotometer (Thermo Scientific). Injections into the dorsal part of the thorax of *B. anynana* larvae were performed under a dissection microscope using 5 µl syringe (Hamilton). We injected 3 and 5 µl of dsRNA at concentration 366 ng/µl into 4th (N = 13) and 5th (N = 10) instar larvae, respectively. Three days later, each individual was injected again with 5 µl of *Antp* dsRNA.

A transcription-blocking vivo-morpholino (5'-AGGCCCAAAGGGATCTGAGAAACTC) for the *B. anynana Dll* gene (AF404825.1) was obtained from GeneTools, LLC (OR, USA). This morpholino is complementary to a 25 bp sequence situated 45 bp upstream of the start-codon in the *Dll* mRNA. Upon binding to its target sequence, it should block the translation and thus prevent the synthesis of Dll protein. The morpholino was diluted in sterile water [1:5] to obtain 0.1 nmole/µl solution, and delivered into larvae in two ways – via injections in the heamolymph or via food. Injections were done in in the dorsal part of the thorax, one day before or after the last larval molt (here referred to as 4th and 5th instar, respectively). Fourth instar larvae were injected with 1.5 – 2.5 µl (N = 40); last instars with 2.5 - 3.0 µl on the first and the third day (N = 20). Similar volumes of sterile water were injected as a control.

For the delivery of the morpholino via larval food, 1% of Tween was added to the morpholino 0.1 nmole/µl solution, 10 µl of which was then applied on maize leaf pieces of approximately 4 cm length. Newly molted 5th instar larvae (N = 25) were placed in small petri dishes with treated maize leaves and kept there until all leaves were eaten. The treatment was repeated two and seven days later. As a control, maize leaves covered with 1% Tween in water were fed to larvae (N = 25) in a similar way. Larvae from all experiments were reared through to adulthood and the eclosed butterflies were examined for morphological aberrations.

RESULTS AND DISCUSSION

B. anynana Antp and *hh* homologues

Sequence analysis of the 450-bp fragment of *B. anynana Antp* obtained in this study revealed that this partial cds encodes 150 amino acids (AA) and shares 95% AA identity with Antp protein of another lepidopteran, the silkworm *Bombyx mori*. It also shows a high degree of similarity to its homologs in other insects, mainly for the homeobox domain (Fig. 1). A 339-bp fragment of *B. anynana hh* was extended to 548 bp in 5'direction. The corresponding 182 AA product is closely similar to Hh proteins of other insects, and shares 93% AA identity with Hh of *J. coenia*, another Nymphalid butterfly (Fig. 2). The fragments obtained in this study thus represent *B. anynana* orthologues of *Antp* and *hh* genes.

a

GTGCCGGGCAGTCCGCCCCTGGAGCAAGCTCAGCAGATGCCGCACCACATGCACCCGCAGCAGCACATGC
AGCACGGCATGCCACCGCACCAGCAGCACGTCATGTACCCGGTGGACGACATGCAGCACCAGACGCAGAT
GCCGCCCATGCACCAGCAGTCCATGCACCCGCAGCAGGCGCCGCCTCAACAACCCCCCGCCGAATACGAAC
GCGTCGCTCCCCAGTCCACTGTACCCTTGGATACGAAGTCAATTTGAACGAAAGCGGGGACGGCAAACGTA
CACCCGGTACCAGACCCTCGAGTTGGAGAAGGAGTTCCACTTCAACCGATACCTGACGCGGAGAAGACGG
ATCGAGATCGCGCACGCCCTCTGTCTCACCGAGCGCCAAATCAAGATCTGGTTCCAGAACCGGCGCATGAA
GTGGAAAAAGGAGAACAAGACCAAGGGC

b

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D.melanogaster          EGGSPPLVDQMSGHHMNAQMTLPHHMGHPQAQLGYTDVGVP---DVTEVHQNHHNM 228
D.erecta                EGGSPPLVDQMSGHHMNAQMTLPHHMGHPQAQLGYTDVGVP---DVTEVHQNHHNM 228
B.anynana               VPGSPPLEQAQQMP----------HHMHP--QQHM-QHGMP---PHQQHVMYPVDD 40
B.mori                  VPGSPPLEQAQQMP----------HHMHP--QQHMVQHGVP---PHQQHLMYPVDD 131
T.castaneum             PTGSPPLTTQSMNN----------HHMGHHMQEQHPQHHQP---HHQQ------QH 189
A.mellifera             GPDSPPLVESQMHHQM--------HTQHPHMQPQQGQHQSQ---AQQQHLQAHEQH 201
C.quinquefasciatus      ENGSPPLEQMGHHMN---------TAQMTIPQHHMGHAQG-------QVHQNPQHM 221
A.aegypti               ENGSPPLEQMGHHMN---------TAQMTIPQHHMGHAQGQDCFPPDQVHQNPQHM 185
                          .****                       *    .          :      .

D.melanogaster          GMYQQQSGVPPVGAPPQGMMHQGQGPPQMHQGHPGQHTPPS--------QNPNSQSSGMP 280
D.erecta                GMYQQQSGVPPVGAPPQGMMHQGQGPPQMHQGHPGQHTPPS--------QNPNSQSSGMP 280
B.anynana               MQHQTQM--PPMHQQSM----HPQQAPPQQ--------------------PPPNTNASLP 74
B.mori                  MQHQTQM--PPMHQQSM----HAQQPPPQQ--------------------PPPNTKPSLP 165
T.castaneum             MMYGGQQ-GANMHQQGP----PHQQPPIQQQPNQGQ-------------QPPGNTAAALP 231
A.mellifera             MMYQQQQQSQAASQQSQ----PGMHPRQQQQAQQHQGVVTSPLSQQQQAAPQGAASANLP 257
C.quinquefasciatus      GMYTNTGGGPPGVVPQQPPNMMHQQPPQLHQGQQPP-------------PNSQNSNSGLQ 268
A.aegypti               GMYTNTGGGPPGVVPQQPPNMMHQQPPQLHQGQQPP-------------PNSQNSNSGLQ 232
                          :                       .   :                        . :

D.melanogaster          SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 340
D.erecta                SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 340
B.anynana               SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 134
B.mori                  SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 225
T.castaneum             SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 291
A.mellifera             SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHYNRYLTRRRRIEIAHALCLTERQIKIW 317
C.quinquefasciatus      SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 328
A.aegypti               SPLYPWMRSQFERKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIW 292
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D.melanogaster          FQNRRMKWKKENKTKG 356
D.erecta                FQNRRMKWKKENKTKG 356
B.anynana               FQNRRMKWKKENKTKG 150
B.mori                  FQNRRMKWKKENKTKG 241
T.castaneum             FQNRRMKWKKENKTKG 307
A.mellifera             FQNRRMKWKKENKSKG 334
C.quinquefasciatus      FQNRRMKWKKENKTKG 344
A.aegypti               FQNRRMKWKKENKTKG 308
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Figure 1. *B. anynana Antp* homologue.

a. Partial cds (450 bp) obtained by degenerate and 5'RACE PCR. **b.** A CLUSTAL W multiple sequence alignment of the predicted *B. anynana* Antp amino acid sequence with the corresponding fragments of Antp proteins of other insects. Numbers on the right show sequence position of amino acids. Sequence identities are marked with (*), conserved substitutions with (:) and semi-conserved substitutions with (.) (*cf.* ClustalW programme). The following protein sequences [GenBank accession numbers] were used: *Bombyx mori* [NP_001037319.1], *Drosophila melanogaster* [NP_996171.1], *Drosophila erecta* [XP_001979126.1], *Tribolium castaneum* [NP_001034505.1], *Apis mellifera* [NP_001011571.1], *Culex quinquefasciatus* [XP_001869455.1], *Aedes aegypti* [XP_001660496.1]. The grey box corresponds to the homeobox region, and underlined amino acids – to sites of degenerate primer design.

a

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ACGCGGGGGGCCGCGGCTTCACCCACCGCCACGGACCGCGCCGCATGACCCCTCTCGTCTTCAACCAGC
ACGAGCCCAACATCAGTGAGAATTCCAAATCCGCCAGTGGCCCTCCCGAGGGCCGCATCACGAGGGAGG
ACGAAAAGTTCAAAGACTTAGTGCCCAATTATAACCCGGACATAGAGTTTAAGGATGACGAGGGCACCGGA
GCCGACCGCCACATGACACAGCGGTGCAAAGAGAAGTTGAACACGCTCGCCATCAGTGTGATGAACCAGT
GGCCCGGGGTTCGACTCCGAGTCATCGAGGGCTGGGACGAGGAGAACTCGGCTCATCTAGAAAACTCAC
TGCACTACGAGGGCCGGGCAGTGGACATCACCACCAGCGACCGGGATCGCAGCAAGTACGGCATGCTGG
CACGCCTTGCTGTGGAAGCCGACTTCGACTGGGTGTTCTATGAGAGCCGGTCCTACATACATTGTTCTGTC
AAGACAGAATCATCAGTGGGCACTGGAGCTGGTTGTTTTCCTTCTGGGTCTGTTGTACAC
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b

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T.castaneum      GPGRGVGRRRGPRKLTPLVFKQHVPNVPENTLTASGLTEGRIGRNDSRFKDLVPNYNQDI  83
N.vitripennis    GPGRGGGRRPILRKLTPLVFKQHVPNVSENTLPASGLGEGRVSRNDKRFRDLVPNYNVDI  101
B.anynana        AGGRGFTHRHGPRRMTPLVFNQHEPNISENSKSASGPPEGRITREDEKFKDLVPNYNPDI  60
J.coenia         ------------------------------KSASGPPEGRITRDDEKFRDLVPNYNPDI  29
A.aegypti        GPGRGIGGPRRTRKLLPLVFKQHVPNVSENSLGASGMQEGPISRNDSKFRNLETNYNKDI  98
D.melanogaster   GPGRGLG-RHRARNLYPLVLKQTIPNLSEYTNSASGPLEGVIRRDSPKFKDLVPNYNRDI  144
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T.castaneum      VFKDEEGTGADRLMTQRCKEKLNTLAISVMNQWPGVRLLVTEGWDEE-GYHTPESLHYEG  142
N.vitripennis    IFKDEEGTGADRLMTQRCKEKLNTLAISVMNQWPGVKLRVTEGWDEE-GKHATDSLHYEG  160
B.anynana        EFKDDEGTGADRHMTQRCKEKLNTLAISVMNQWPGVRLRVIEGWDEENSAHLENSLHYEG  85
J.coenia         DFKDDEGTGADRLMTQRCKEKLNTLAISVMNQWPGVRLRVIEGWDEE-NSHLDNSLHYEG  88
A.aegypti        IFKDEEGTGADRVMTQRCKEKLNILAVSVMNQWPGLRLMVTEGWDED-HMHARESLHYEG  157
D.melanogaster   LFRDEEGTGADRLMSKRCKEKLNVLAYSVMNEWPGIRLLVTESWDED-YHHGQESLHYEG  203
                  *:*:******* *::******* ** ****:***::* * *.***:   *  :******

T.castaneum      RAVDITTSDRDRSKYGMLARLAVEAGFDWVYYESRAHIHCSVKSESSQAAKYGGCFSGESTVL  205
N.vitripennis    RAVDVTTSDRDRAKYGMLARLAVEAGFDWVYYESRSHIHCSVKSESSSAGKSGGCFPGASLVR  223
B.anynana        RAVDITTSDRDRSKYGMLARLAVEADFDWVFYESRSYIHCSVKTESS-VGTGAGCFPSGSVVH  182
J.coenia         RAVDLTTSDRDHSKNGMLARLAVEAGFDWVFYENRSYIHCSVKTESS-VGTGAGCFPSGAVVH  150
A.aegypti        RAVDIMTSDKDRSKIGMLARLAVEAGFDWVYYESRSHIHCSVKSDSSQSNHASGCFTGDSTVQ  220
D.melanogaster   RAVTIATSDRDQSKYGMLARLAVEAGFDWVSYVSRRHIYCSVKSDSSISSHVHGCFTPESTAL  266
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Figure 2. *B. anynana hh* homologue.
a. Partial cds (548 bp) obtained by 5'RACE PCR. **b.** A CLUSTAL W multiple sequence alignment of the predicted *B. anynana* Hh amino acid sequence with the corresponding fragments of Hh proteins of other insects. Numbers on the right show sequence position of amino acids. Sequence identities are marked with (*), conserved substitutions with (:) and semi-conserved substitutions with (.) (*cf.* ClustalW programme). The following protein sequences [GenBank accession numbers] were used: *Junonia coenia* [AAD08931.1], *Drosophila melanogaster* [AAA16458.1], *Tribolium castaneum* [NP_001107837.1], *Aedes aegypti* [XP_001657979.1], *Nasonia vitripennis* [XP_001605475.1].

No evidence for Hh signalling in eyespot centres in *B. anynana* larval wings

In situ hybridizations were performed to determine the spatial distribution of *hh* and *ptc* transcripts in the wing discs of *B. anynana* WT larvae. As in *D. melanogaster* (Lee *et al*. 1992) and other insects including the butterfly *J. coenia* (Keys *et al*. 1999), *hh* mRNA was detected in the posterior compartments of all 44 early and mid-fifth instar wings examined (Fig. 3b), and in 32 out of 48 wings of late-fifth instars (Fig. 3c). Detection of *hh* mRNA presumably failed in the other 16 wings which were covered by rigid peripodial membrane that may not be permeable to the probe. Transcript of the Hh receptor-encoding gene *ptc* was found along the antero-posterior compartment boundary and in vein lacunae in all

50 mid- and late-fifth instar larvae wing discs examined (Fig. 3e), also resembling patterns found in *D. melanogaster* (Phillips *et al*. 1990) and *J. coenia* (Keys *et al*. 1999). Higher levels of *ptc* mRNA were only occasionally detected in the regions corresponding to centres of some eyespots (Fig. 3f), but *hh* transcripts were never seen in the cells flanking eyespot foci. Stainings with control sense probes produced no patterns.

The absence of *hh* transcripts in eyespots of *B. anynana* prevented us from testing the predictions of models proposed by Marcus & Evans (2008) for eyespot focus determination. We found that expression patterns of Hh pathways genes differed in some aspects from those described for larval wings of *J. coenia*, where *hh* transcripts were found around, and *ptc* mRNA in the regions corresponding to future eyespot foci (Keys *et al*. 1999). The conserved function of Hh signaling in insect wing (Tabata & Kornberg 1994) was evident in both species, as was clear from *hh* upregulation in the posterior compartment and high levels of *ptc* transcription in the cells just anterior to the antero-posterior boundary. However, the putative role for this pathway in eyespot focus determination as suggested by studies in *J. coenia* was not confirmed in *B. anynana*. Both Hh signal transducer Ci and its target En were detected in eyespot foci of *J. coenia* (Keys *et al*. 1999; Reed, Chen & Nijhout 2007), and in all eyespot foci of *B. anynana* (Fig. 3d; see also Keys *et al*. 1999). The absence of the Hh ligand and its receptor in the eyespot field in one of the species is thus very remarkable, and suggests that activation of *Ci* and *en* in eyespot foci might be Hh-independent in *B. anynana*. The genetic pathways underlying eyespot development in these nymphalids may differ substantially. This emphasizes the necessity of studies of complete genetic pathways in multiple organisms, both at the levels of gene expression and of gene function.

Wg as a putative morphogen: sense and antisense transcripts in pupal wings

The spatial patterns of *wg* mRNA were examined in pupal wings at 12 – 18 hours post pupation (hpp, N = 60). In the hindwings, *wg* transcripts were detected in eyespot foci in pupal wings at 14 – 18 hpp (Fig. 4b,d). Unexpectedly, stainings with the control sense probe on the opposite side wings of the same individuals produced similar results (Fig. 4c,d). To check for the possibility that these patterns are due to unspecific binding of the sense probe, we performed *in situ* hybridizations in larval wings. Consistent with previous findings (Carroll *et al*. 1994), *wg* mRNA was detected in the wing margin, and only with the anti-sense probe (Fig. 4i,j). This suggests that the control probe hybridizes in a specific fashion to transcripts complementary to *wg* mRNA, which are present in pupal, but not in larval wings. Expression patterns of *wg* sense and anti-sense transcripts in the forewings were more complex than those observed in the hindwings. At 14

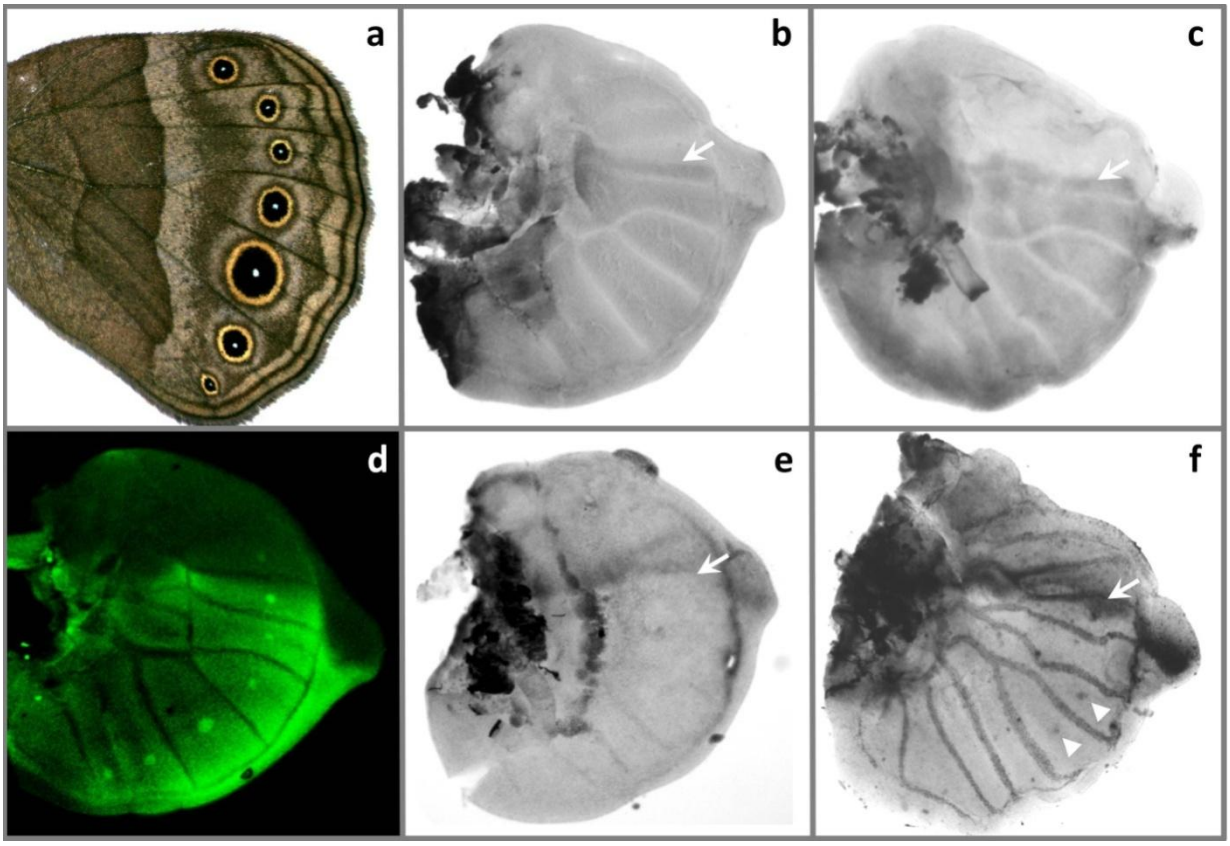

Figure 3. Expression patterns of *hh*, *ptc* and *en* in larval wing discs.
a. Representative image of *B. anynana* hindwing with seven marginal eyespots. **b.** *hh* mRNA is detected in the posterior wing compartment in mid-5th instar larval wings (stage 1, arrows point to the antero-posterior boundary) and **c.** in late 5th instar larval wings (stage 2.5). **d.** *en* is upregulated in the posterior compartment and in the centres of all eyespots. **e.** *ptc* is expressed along the antero-posterior boundary (arrows) in mid-5th instar wings, and also in some eyespot foci (arrowheads) in the late 5th instar wing discs (**f**).

hpp, both *wg* mRNA and its complementary transcript were detected in eyespot foci (Fig. 4e). In older pupal wings (15 - 18 hpp), additional ring-like patterns of *wg* mRNA, but not of the anti-sense transcript, were found around forewing eyespot foci at a time when both had already disappeared from the centres of posterior forewing eyespots (Fig. 4f-h).

These results are consistent with findings of Monteiro and colleagues (2006) who detected Wg protein in eyespot foci at 10 – 16 hpp. They suggest that Wg may indeed be (one of) the morphogen(s) produced in eyespot centres. However, *wg* mRNA processing, and thus protein levels, might be modulated by its anti-sense transcript in a highly specific way. Recent findings have revealed that expression of complementary anti-sense transcripts in a tissue-specific manner is a widespread phenomenon, and that they not only regulate the expression levels and processing of the sense transcripts, but can also silence their transcription (Lapidot & Pilpel 2006; Werner & Sayer 2009). Anti-sense transcripts may, therefore, be essential to the fine-tuning of specific genes. It is unclear if both *wg* transcripts are expressed in exactly the same cells in *B. anynana* pupal wings, and how they interact, but it is possible that translation

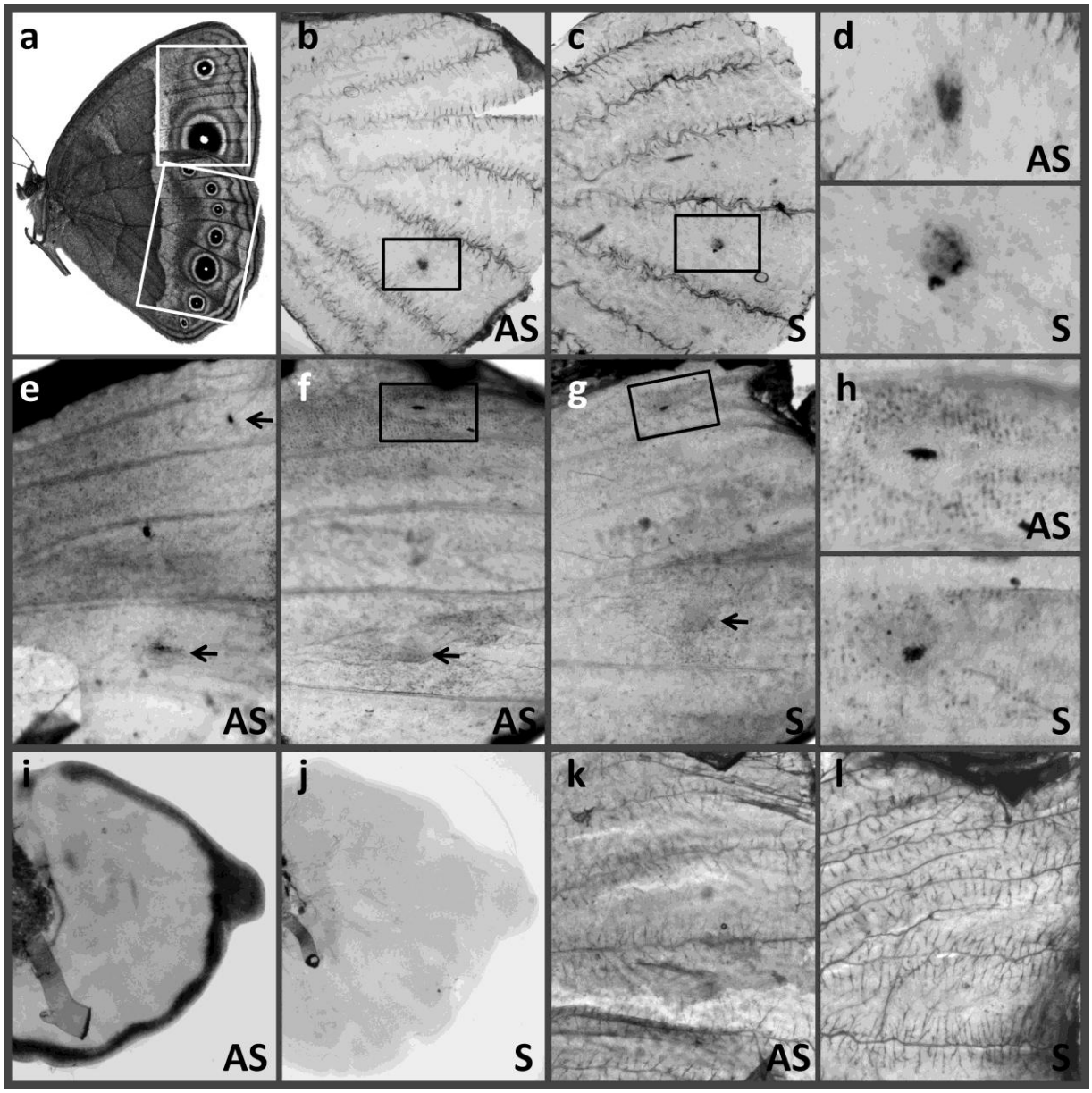

Figure 4. Expression patterns of *wg* and *fz*.
a. Ventral side of a *B. anynana* wild-type female; white squares indicate sections of the wings shown in panels **b**, **c**, **e** – **g**, **k**, and **l**. Pupal hindwings stained with *wg* anti-sense (**b**) and sense (**c**) probes at 14 – 18 hpp show similar patterns in eyespot foci (enlargement of 5th hindwing eyespot in **d**). In early pupal forewings (14 hpp), *wg* sense (**e**) and anti-sense transcripts (pattern identical to that in **e**) were detected in eyespot foci (arrows). At later stage (15 – 18 hpp) *wg* mRNA (**f**) was found in and around anterior eyespot focus, but not in posterior eyespot focus (arrows). Anti-sense transcripts (**g**) were detected in anterior eyespot foci, but not in a surrounding ring (enlargement of the anterior eyespot area in **h**). Control stainings in last instar larval wing discs produced a pattern in the distal margin with anti-sense probe (**i**), but not with sense probe (**j**). Visualization of *fz* produced similar patterns with anti-sense (**k**) and sense probes (**l**).

from the sense strand starts slightly earlier than that from the complementary strand. This might be sufficient to produce Wg protein, and could be involved in controlling its levels in a very precise manner.

Analysis of a signal transduction pathway requires examination of all its components (*i.e.* ligand, receptors etc.) in an integrated way. *In situ* hybridizations with probes against the *B. anynana* homologue of *fz*, a gene

encoding one of the Wg receptors (Bhanot *et al*. 1999), produced identical patterns in all pupal wings examined (N = 30; Fig. 4k,l). *fz* mRNA and the anti-sense transcripts were detected throughout the wing epidermis at very low levels, but the distribution of these transcripts was seemingly not cellular. This suggests that *B. anynana fz* gene is not expressed in early pupal wings or is expressed at very low levels (as in *Drosophila*, Park *et al*. 1994), and that it is not essential for Wg signal transduction in this type of tissue. Another receptor of this family might fulfill this role (*e.g.* four Frizzled proteins have been identified in flies, two of which are redundant, see Bhat 1998; Bhanot *et al*. 1999).

Antp is associated with position, number and shape of eyespot foci

The spatial patterns of *Antp* expression, as examined in the wing discs of 'wild-type'*B. anynana* final instar larvae with antibodies 4C3 and 8C11 (Fig. 5b,e) and with the riboprobe against mRNA (Fig. 5i), produced similar patterns and revealed a strong association between the position and number of future eyespot centres, and regions with upregulated levels of this Hox gene. Both mRNA and protein were detected in the presumptive centres of the seven hindwing eyespots, and in all four potential eyespot foci on the forewing. Usually, *B. anynana* forewings bear two eyespots (Fig. 5a), but extra eyespots in the intermediate positions are typical for the *Spotty* mutant (Brakefield & French 1993) and are found occasionally in 'wild-type'stock butterflies and in lines selected for large eyespots (Monteiro, Brakefield & French 1994; Beldade & Brakefield 2003). Upregulated levels of *Antp* may indicate wing positions which are competent to produce eyespots, but not necessarily doing that. Moreover, expression pattern of this Hox gene perfectly correlated with the shape and position of the single elliptical eyespot in larval hindwings of the venation mutant Cyclops (Fig. 5g,h).

Stainings with the anti-Ubx FP6.87 antibody showed high levels of expression of the target gene in the hindwing, but not in the forewing tissue (Fig. 5c,f). This was consistent with the previously described function of Ubx in determination of dorsal appendages on the third thoracic segment (Weatherbee *et al*. 1999; Tomoyasu, Wheeler & Denell 2005). We found no clear evidence for the upregulation of this Hox gene in the presumptive eyespot fields in larval wings of *B. anynana*. In 5 out of 30 individuals that were examined, the antibody was, however, detected at slightly higher levels in the centres of future eyespots. It is not impossible that this antibody binds unspecifically to Antp protein, which is present in eyespot foci at high levels. Since this anti-Ubx antibody recognizes the homeodomain bearing region of both Ubx and Abdominal-A proteins in *Drosophila* (Kelsh *et al*. 1994) it might weakly bind to the equivalent region of Antp and produce faint patterns in the eyespot regions.

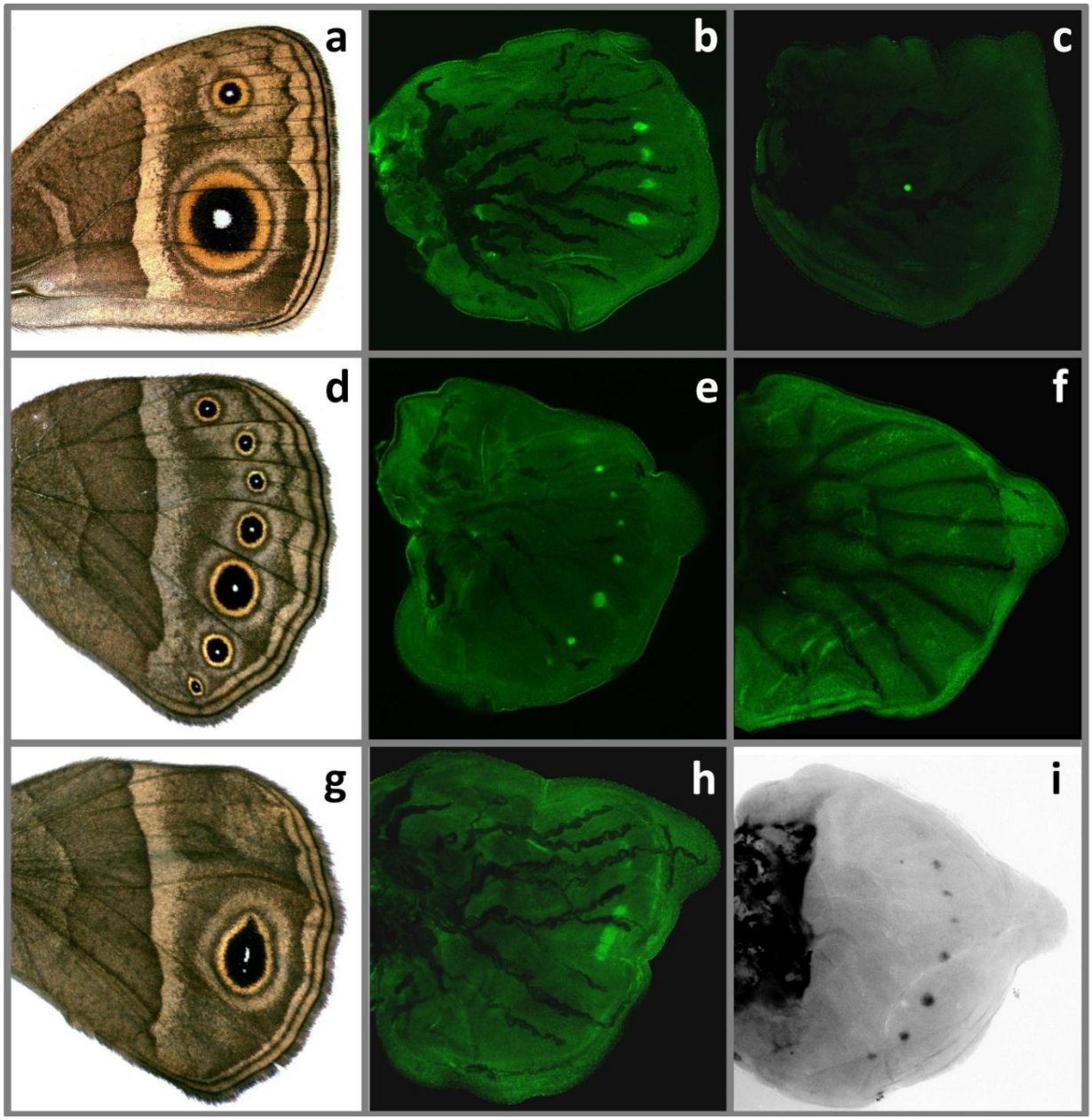

Figure 5. Expression patterns of the Hox genes *Antp* and *Ubx* in larval wings. Representative images of adult *B. anynana* **a.** forewing and **d.** hindwing. In wild-type larvae, Antp protein was detected in eyespot foci (**b** and **e**), while Ubx was absent from the forewing (**c**) and expressed ubiquitously in the hindwing (**f**). In the *Cyclops* mutant, typically one elongated eyespot is present on the adult hindwing (**g**), and Antp is found in a stripe that corresponds to a single eyespot focus (**h**). Patterns of *Antp* mRNA detected with the riboprobe (**i**) were similar to those detected with the antibodies (compare to **e**).

Antp upregulation is the earliest event in the process of eyespot development

Stainings with the 4C3 antibody in the wing discs of young last instar larvae (1 – 2 days after the last molt; wing developmental stage 0 - 1) revealed that upregulation of this Hox gene in eyespot centres occurs very early in the final instar before the trachea become extended in the vein lacunae (stage 0.5, Fig. 6). In 21 out of 26 larvae examined at this stage, Antp protein was already visible in eyespot centres. Vein lacunae and short tracheoles were discernable in all these wings. The finding that *Antp* is upregulated early in eyespot focal cells suggests that it may be involved in the process of focus determination.

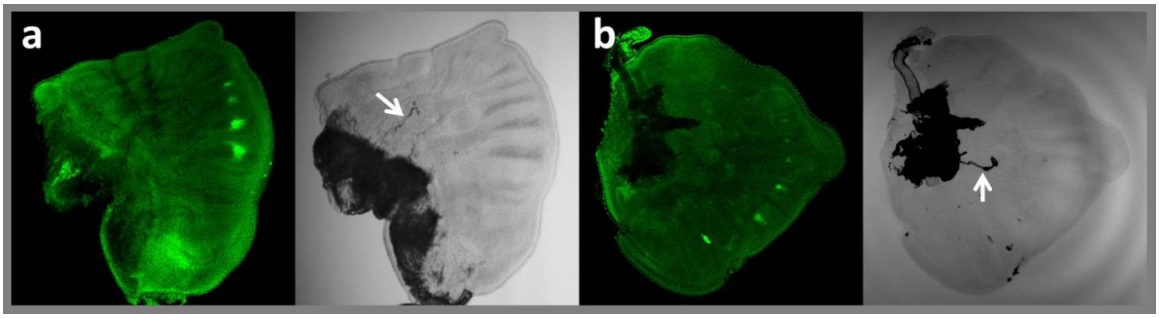

Figure 6. Expression patterns of *Antp* at early larval stages.
Representative images of **a.** a forewing (left – immunostaining with 4C3 antibody, right – light microscopy image of the same wing) and **b.** a hindwing at stage 0.5, characterized by presence of few discernable vein lacunae and small tracheoles (arrows) extending from basal tracheal mass. At this stage, Antp protein is already detected in four eyespot centres on the forewing, and in most eyespot foci on the hindwing.

To establish the relationship between the spatio-temporal expression of *Antp* and other genes previously implicated in eyespot determination (see Chapter 1), we produced co-stainings with antibodies that recognize Sal, N and Dll proteins (Fig. 7). Expression patterns of these genes in final-instar wing discs were consistent with those described previously for *B. anynana* (Carroll *et al*. 1994; Monteiro *et al*. 2006) and *J. coenia* (Reed, Chen & Nijhout 2007). Namely, N and Dll proteins were detected in the intervein stripes in the early last-instar wings, before tracheal extension (stages 0.5 – 1), and became concentrated in discrete focal patterns at later stages. Just as in *J. coenia*, Sal was initially upregulated in broad intervenous bands, and subsequently became expressed only in the focal cells of the future eyespots. We found that *Antp* upregulation in eyespot foci preceded that of N, which has been described previously as the earliest known event in the process of eyespot determination (Reed & Serfas 2004). At stage 0.5, Antp was already present in four eyespot centres on the forewing and in 5-7 of the hindwing eyespot foci, while N was expressed broadly in intervenous regions without any noticable upregulation in the presumptive focal cells (Fig. 7). During the following stages, *Antp* became strongly upregulated in the centres of those wing cells that will produce eyespots (*i.e.* two on the forewing and seven on the hindwing) and faded in the intermediate forewing cells.

Our analysis of *Antp* expression in *B. anynana* larval wings revealed that the upregulation of this Hox gene in eyespot centres is the earliest event associated with eyespot development described to date. Not only does Antp appear in eyespot foci before N, Dll or Sal, but it is also upregulated in discrete focal patterns directly, while other genes are also expressed in 'non-eyespot' areas, such as wing margin and intervein bands (Fig. 7). How can this result be integrated with the current knowledge about genetic mechanisms of eyespot focus determination? In the model that takes into account expression pattern data from *B. anynana* and *J. coenia*, and the knowledge of gene interactions from *D.*

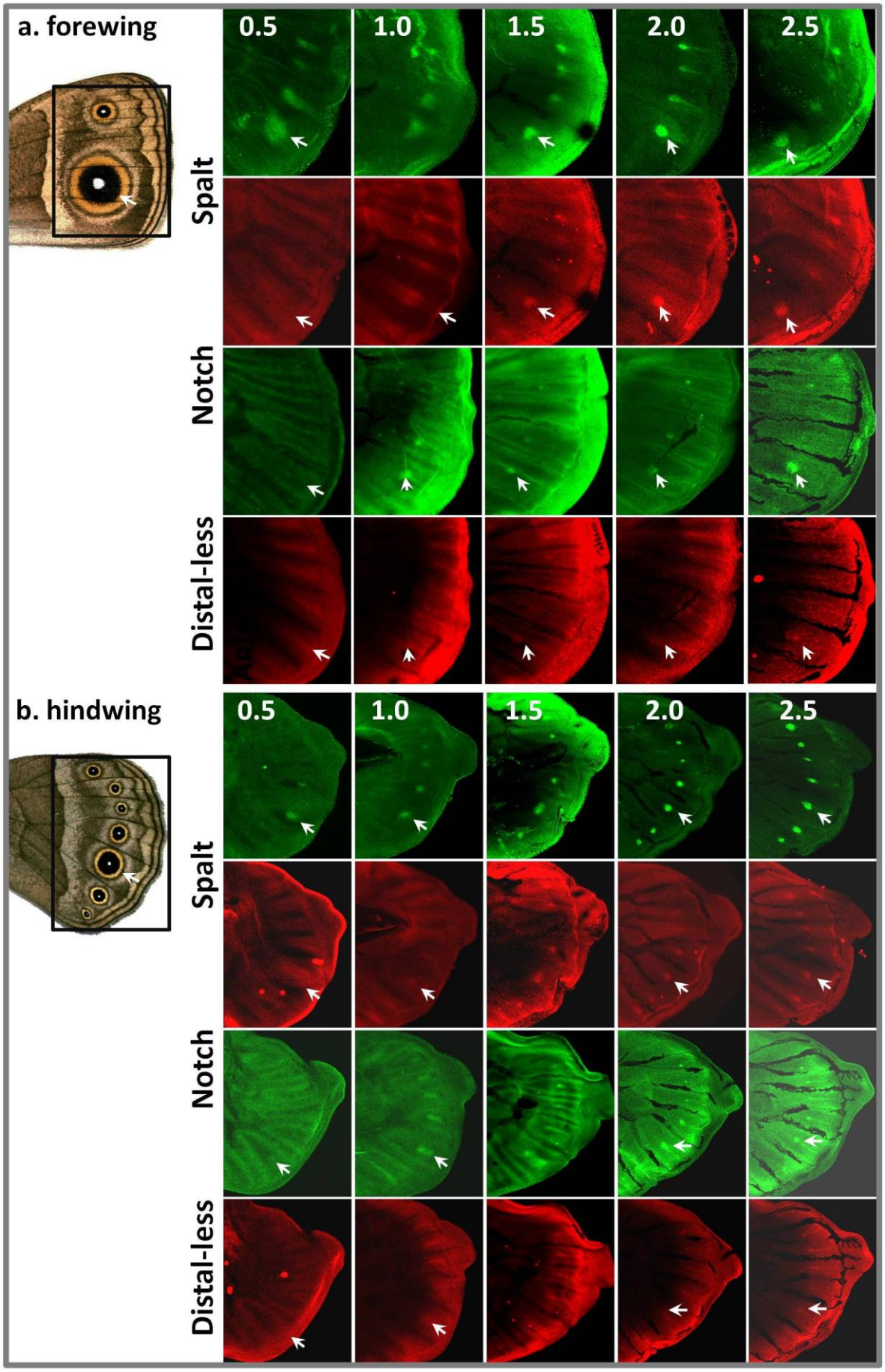

Figure 7. Spatio-temporal patterns of expression of *Antp*, *sal*, *N* and *Dll* in larval wings. Representative images of forewing (**a**) and hindwing (**b**) sections are shown at wing developmental stages 0.5 - 2.5 (arrows point to the eyespot indicated on the adult wing images). Upregulation of *Antp* in eyespot centres is followed by that of *sal*, *N* and *Dll*.

melanogaster, eyespot formation is initiated in the areas of wing epidermis with the lowest levels of N downregulation by unknown repressors diffusing from the wing margin and veins (Evans & Marcus 2006). Stable levels of N in presumptive eyespot focal cells lead to upregulation of *Dll* and, subsequently, to activation of other 'eyespot' genes. We propose that a similar mechanism (*i.e.* diffusion of wing vein/margin signalling molecules) might exist that activates expression of *Antp* in a subset of wing cells very early during wing development, and that Antp and N control eyespot focus determination in *B. anynana*. Antp is one of the highly conserved Hox proteins required for specification of embryonic segment and appendage identity (Hughes & Kaufman 2002). It determines the identity of thoracic segments (Struhl 1982; Schneuwly, Klemenz & Gehring 1987) during *Drosophila* embryogenesis. It is also expressed in the leg discs during the larval stage where it represses genes required for antennal development (Casares & Mann 1998), thereby acting as the key selector gene responsible for leg identity (Emerald & Cohen 2004). Antp is also involved in insect haematopoiesis (Crozatier & Meister 2007) and nervous system development (Sprecher et al. 2004; Rogulja-Ortmann, Renner & Technau 2008), and in silk gland development in the silkworm *Bombyx mori* (Nagata *et al*. 1996). Interestingly, ectopic expression of *Antp* combined with activation of N signaling is capable of inducing leg and wing development via upregulation of the genes *Dll* and *vestigial* in *D. melanogaster* (Kurata *et al*. 2000). It is possible that a similar type of interaction between Antp, N and Dll takes place in the developing *B. anynana* wings leading to initiation of the eyespot-inducing cascade. This study represents the first finding of a role of Antp in the development of adult colour patterns. If functional tests could confirm that this Hox gene regulates eyespot determination in butterfly wings, it may become the first example of its novel, lineage-specific regulatory function.

No visible effects of *Dll* and *Antp* knock-down on adult morphology

To establish a functional relationship between eyespot determination and upregulation of *Antp* and *Dll* in eyespot foci, we attempted to prevent the translation of these genes in the developing larval wings by means of RNA interference (RNAi) with dsRNA against *Antp* mRNA, or translation-blocking *Dll* vivo-morpholino. In total, we injected between 2.9 and 3.7 µg of *Antp* dsRNA in the late 4th and early 5th instar larvae, respectively. All 23 injected animals developed normally, and no effect on eyespot pattern or any other aspect of morphology was observed in the adults. Feeding of larvae with *Dll* morpholino (3 nmole per individual, N = 25), as well as injections in the 4th instar larvae (0.15 nmole per individual, N = 40), did not have any noticeable effect on developmental time, morphology or wing colour pattern. Injections in the early

5th instars (0.6 nmole per individual, N = 20) did not produce any obvious effects on leg or wing morphology, or on eyespot pattern, except shortened antennae in two individuals.

It is likely that both injections of dsRNA and morpholino, as well as application of the latter via food, failed to inhibit the translation of the target genes. Unfortunately, very little succes has been achieved with RNAi in Lepidoptera, in particular with the attempts to downregulate genes involved in wing patterning by means of dsRNA injections (see Monteiro & Prudic 2010, but also Masumoto, Yaginuma & Niimi 2009), and the reasons for this are unclear. Moreover, morpholinos have been widely and succesfully applied in vertebrate research, but only a few studies were published that made use of morpholino-mediated inhibition of gene translation in arthropods, mostly during embryogenesis (*e.g.* Bucher & Klinger 2004; Ozhan-Kizil, Havemann & Gerberding 2009). In the future, a transgenic approach might be applied more successfully to functional studies of candidate genes for eyespot formation (Ramos & Monteiro 2007), and on the dissection of molecular and genetic mechanisms underlying co-option of such conserved genes in the evolution of novel traits.

CONCLUDING REMARKS

We examined the spatio-temporal expression patterns of genes encoding Wg and Hh signaling molecules, their respective receptors Fz and Ptc, and the Hox proteins Antp and Ubx in the developing wings of *B. anynana* butterflies. Expression of *wg* during the signaling stage of eyespot morphogenesis was consistent with its role as eyespot morphogen (Monteiro *et al*. 2006), but also suggested some unexpected aspects of its potential regulation by anti-sense transcripts. Our study also implicated Antp in eyespot determination and provided the first evidence for a Hox protein being associated with an adult insect colour pattern element. Moreover, it revealed unforeseen differences in *hh* and *ptc* expression patterns between *B. anynana* and *J. coenia* butterflies and suggested that the genetic mechanisms underlying nymphalid eyespot formation might have diversified substantially. Thus, the evolution of eyespot development may have led to a surprising level of variability in underlying molecular mechanisms, as the ecological potentials of such novelties were exploited in morphological and behavioural diversification. These results emphasize the importance of a comparative analysis of eyespot development in a broad range of butterfly species. They also make it obvious that functional analysis of candidate genes, expressed in suggestive patterns during eyespot formation, is a critical step in the study of eyespot evolution. Since a series of studies have suggested an

association of increasing numbers of genes with the determination of adult colour pattern, it is crucial to confirm that these genes are indeed involved in pattern formation. Only then will it be possible to explore how the gene networks generating eyespot patterns have originated and evolved. Unfortunately, our knock-down experiments for two such genes failed to establish a relationship between observed expression patterns and eyespot phenotype.

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